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(71) Applicant (for all designated States except US): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 9500 Gilman Drive, Mail Code 0910, La Jolla, CA 92037-0910 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **WITZTUM, Joseph** [US/US]; 6912 Ofria Court, San Diego, CA 92120 (US).

**TSIMIKAS, Sotirios** [US/US]; 2802 Arnoldson Avenue, San Diego, CA 92122 (US). **SHAW, Peter, X.** [US/US]; 10860 Caminito Arcadia, San Diego, CA 92131 (US). **SILVERMAN, Gregg** [US/US]; 9500 Gilman Drive, Mail Code 0613-C, La Jolla, CA 92037 (US). **PALINSKI, Wulf** [US/US]; Apt. 20, 4060 Porte De Palmas, San Diego, CA 92122 (US).

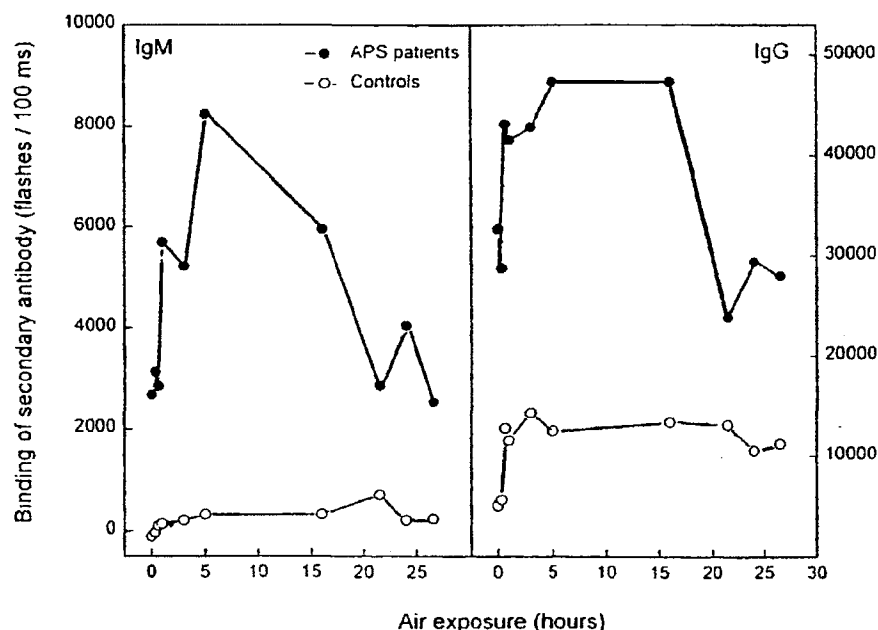
(74) Agents: **MCCLAIN, James, W.** et al.; Brown, Martin, Haller & McClain LLP, 1660 Union Street, San Diego, CA 92101 (US).

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(54) Title: STANDARDIZED OXIDIZED LDL ASSAY



(57) Abstract: The oxidation of LDL plays an important role in the development of the pathology of atherosclerosis. The invention is a method to standardize an assay for the presence of OxLDL in patient serum by the use of phosphorocholine (PC), a readily available, highly stable, small molecule standard. PC, alone or linked to a carrier protein, may be used as a competitor or to develop a standard curve in an ELISA assay. Alternatively, PC linked to a carrier protein is bound to a microtiter well and patient sera is assayed for binding to reveal the presence of anti-OxLDL antibodies.

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## **STANDARDIZED OXIDIZED LDL ASSAY**

### **CROSS-REFERENCES TO RELATED APPLICATIONS**

- 5        This application claims the benefit of priority of United States provisional application Serial Number 60/203978 filed May 12, 2000 which is incorporated herein by reference in its entirety.

### **GOVERNMENT INTEREST**

- 10       This invention was made with government support from the National Institutes of Health under grant numbers 5P50-HL59689 and 5RO1-HL57505.

### **BACKGROUND OF THE INVENTION**

- 15       Lipoproteins are primary carriers of plasma cholesterol and triglycerides. They are micellular lipid-protein complexes that contain proteins (referred to as apoproteins) and polar lipids organized in a surface film that surrounds a neutral lipid (triglyceride and cholesteryl ester) core. The proteins serve as recognition factors for lipoprotein receptors. For example, a species of apolipoprotein B100 (apo B100) which is synthesized in the liver  
20       where low density lipoprotein particles (LDLs) are assembled, is recognized by cellular LDL receptors. By recognizing apo B100, these receptors bind LDL particles and extract them from the plasma. The LDL is thereby taken into the cell and broken down, yielding cholesterol and lipids to serve the cell's needs. This uptake is controlled by downregulation of LDL receptors on  
25       the cell surface to maintain an appropriate balance of lipids and cholesterol with other cellular components.

- In the arterial wall, as well as other sites, LDL can become damaged by oxidation to yield oxidized LDL (OxLDL). OxLDL are no longer taken up by the LDL receptor of normal cells. They are instead recognized by  
30       scavenger receptors on phagocytes which are responsible for clearance of foreign particles from the blood stream and other tissues, such as the arterial wall. However, uptake of OxLDL into these cells is not regulated. As a result, phagocytes become overfilled with the OxLDL lipids, including oxidized lipids, and become foam cells which are characterized by their appearance and  
35       structural instability. They are believed to be "activated," secreting a variety of factors leading to plaque instability and blood coagulation. In addition, the

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death of foam cells can result in the deposition of lipids in the arteries.

Deposition of fats in blood vessels is only one of the factors that contributes to the development of the pathology of atherosclerosis.

Atherosclerosis is a chronic inflammatory disease that results from a complex  
5 interplay of environmental, metabolic and genetic risk factors. Oxidation of  
LDL generates a variety of oxidatively modified lipids and proteins that  
represent highly immunogenic neo-determinants for the immune system. In  
murine models for atherosclerosis, such as ApoE-deficient (ApoE<sup>-/-</sup>) mice,  
atherosclerosis is correlated with development of high titers of "auto"-  
10 antibodies to various "oxidation specific" neo-epitopes of oxidized LDL  
(OxLDL) (Palinski, 1994; Palinski, 1996). The consequences of such cellular  
and humoral responses are still poorly understood, but under certain  
conditions they can clearly modify the natural history of the disease. For  
example, immunization of hypercholesterolemic rabbits or mice with  
15 homologous models of OxLDL ameliorates atherosclerosis (Palinski, 1995;  
Freigang, 1998).

Changes in the oxidation state of LDL are associated with a number of  
disease states including diabetes, renal failure and preeclampsia. Similarly,  
patients frequently have increased levels of OxLDL after heart transplant.  
20 Aldehyde modified LDL (e.g. MDA-LDL) seems to be more strongly  
associated with other disease states including acute myocardial infarction and  
coronary artery disease. Therefore, different types of LDL modification are  
indicative of different disease states.

A number of assay systems have been reported using antibodies to  
25 monitor the levels of both LDL and OxLDL in the blood, and thus  
atherosclerotic disease. Young et al. (US Patent no. 5,460,947) teach the  
use of an immunoassay to detect the amount of apolipoprotein B (apo B) in  
serum. However, such an assay detects both oxidized and non-oxidized  
LDL. Native LDL is a normal transport mechanism for cholesterol and lipids  
30 throughout the body. Such a test would be of limited value as an indicator of  
the development and progression of atherosclerotic disease as compared to  
an assay for OxLDL.

Antibodies specific for OxLDL exist, including a panel of B-cell  
hybridomas generated by fusion of spleen cells of diseased ApoE<sup>-/-</sup> mice that  
35 were not immunized with any antigen with P3 x 63AG8.653.1 myeloma cell  
line (Palinski, 1996, incorporated herein by reference). Reflecting the

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disease-associated immune response seen in humans, 494 of 768 pooled hybridoma wells contained antibodies that bound to one or more epitopes of OxLDL as determined by binding to LDL exposed to a number of different modifying agents (e.g. malondialdehyde (MDA), copper ions, air). Seventeen  
5 hybridoma lines were cloned and termed "E0" antibodies, E01 to E017. All were IgM antibodies that bound strongly either to a model epitope of OxLDL, MDA-modified LDL (MDA-LDL), or to LDL oxidized by exposure to copper (Cu-OxLDL). No antibodies that bound to both MDA-LDL and Cu-OxLDL were found in this panel, suggesting that distinct epitopes were recognized on  
10 differently modified LDLs. Each of the Cu-OxLDL specific antibodies was later shown to bind oxidized phospholipids and specifically to 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine (POVPC), an oxidation product derived from 1-palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphorylcholine (PAPC). Of considerable interest, all of these POVPC-  
15 specific autoantibodies recognized the lipid moiety of OxLDL, as well as the delipidated modified apoB, but not native LDL which contains many non-oxidized phospholipids. Thus these antibodies bind to oxidized phospholipids, or their protein adducts (e.g. with apoB or BSA).

Despite the number of antibodies for OxLDL available, the  
20 development of standard assays for OxLDL has been hindered by lack of a standard control for the assay. Although it is possible to purify LDL for use in a laboratory setting, it is difficult to prepare the large quantities required for clinical assays without some oxidation of the LDL, which would vary from batch to batch. This would result in interassay variation which can be  
25 tolerated and controlled for in a laboratory research setting; however, such variance in a hospital, diagnostic laboratory would be problematic.

This problem is clearly illustrated by an example of an antibody binding assay using an unsaturated phospholipid, cardiolipin (CL). CL dissolved in ethanol was rapidly dried in a microtiter well under nitrogen. The wells were  
30 then exposed to air at room temperature for 0 to 26 hours. Various sera from patients with the antiphospholipid antibody syndrome were diluted and incubated with each well for 1 hour. Wells were washed and bound antibody was detected using an alkaline phosphatase-linked secondary antibody followed by a chemiluminescent substrate. The results are shown in Figure 1  
35 (figure 5B, Horoko et al 1996). Binding of sera to CL peaked at 5-6 hours for IgM and 5-10 hours for IgG. A marked variation in binding to the zero-time

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samples of different phospholipid preparations was also observed between experiments. Even with careful handling, samples become progressively more oxidized through repeated freeze-thaw cycles. These data clearly indicate inaccuracies inherent in system using readily oxidizable substances  
5 as controls in immunoassays.

Similarly, other studies have demonstrated that small changes in the time of exposure of LDL to oxidizing agents can result in large changes in antibody binding. It is known that the extent of copper induced oxidation of LDL has a profound difference on the extent of antibody binding. For  
10 example, binding of antibodies to copper oxidized-LDL increased dramatically due to increasing periods of time of oxidation (Fig. 2) (Palinski et al, 1996).

Holvoet et al. (International Publication WO 98/85248) teach the use of malondialdehyde modified low density lipoprotein (MDA-LDL) as a calibrator or control to determine the amount of OxLDL in patient serum. The  
15 application teaches the use of two antibodies, one that binds both MDA-LDL and OxLDL with high affinity, and a second that binds only MDA-LDL with high affinity, and the use of MDA-LDL prepared in a manner such that the LDL has a defined number of aldehyde modifications. However, LDL cannot be purchased, the purification of LDL is non-trivial, and the potential for  
20 oxidation during purification can be significant. Although the extent of MDA-LDL in patient serum could likely be determined by the assay, there is no way to control for the extent of oxidation of the phospholipids that exist in the LDL particles. Additionally, relatively small changes in the number of aldehyde modifications of on LDL can drastically change the affinity of the antibodies.  
25 A four-fold increase in the number of aldehyde modifications (from 60 to 240) yields a 100-fold increase in the affinity of the antibody for the antigen. Although one would be able to distinguish between MDA-LDL with 60 aldehyde modifications as compared to 240, small differences in variation of the control would be amplified by the properties of the antibody. This is  
30 evident by the interassay variation that they see, 7.6-16.9%, relatively high for a clinical assay. It should also be considered that LDL would never become so extensively modified *in vivo* to contain 60 modified lysines. Upon modification of approximately 18-20 the LDL would be cleared by phagocytes. Therefore, even if the MDA-LDL could be prepared in such a way that it was  
35 stable, it would be difficult to extrapolate the amount of MDA-LDL present with very few modified lysines as compared to a highly modified LDL.

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**SUMMARY OF THE INVENTION**

The invention is a method to standardize an assay for the presence of OxLDL by the use of phosphorylcholine (PC) as a competitor of binding of OxLDL from patient sera or other sources, to E06 or T15 antibody. (In this case, PC does not stand for phosphatidylcholine, an intact phospholipid containing two fatty acid chains.) PC is a relatively small, soluble, commercially available (Sigma Chemical Corp.), small molecule that makes standardization of an antibody assay for OxLDL feasible and reproducible. Because preparation of OxLDL *in vitro* is not reproducible, the availability of a small molecule that is recognized equally well by the monoclonal antibodies E06 and T15 provides ease in standardization.

E06 or T15 antibody can be used to detect the level of OxLDL in serum by ELISA, RIA or other standard immunoassay technique. The E06 and T15 antibodies can be monoclonal or polyclonal antibodies. Alternatively, as the sequence of the light and heavy chains is known, the antibodies may be converted to single chain antibody variable region fragments (scFv) by methods well known to those skilled in the art. The immunoassay can be performed either by first capturing the LDL on a microtiter well by use of an antibody that binds both oxidized and non-oxidized LDL (e.g. anti-apoB), and then detection of the OxLDL by a labeled E06 antibody. Alternatively, E06 antibody can be bound to the bottom of the microtiter well and the amount of OxLDL bound determined by the use of labeled anti-LDL antibody. OxLDL could also be used to coat the microtiter wells and various concentrations of patient sera, putatively containing OxLDL, could be mixed with a constant, limiting amount of labeled (e.g. biotinylated) E06 or T15 to compete for binding to the OxLDL on the plate. For each assay, under standard conditions, a standard curve could be developed using PC as a competing agent. Alternatively, a parallel set of reactions can be run using PC as a source of competing agent rather than patient sera. The PC can be used alone, or linked to a carrier protein, such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH).

PC linked to a carrier can also be used to coat microtiter wells to assay for the presence of antibodies to OxLDL or OxHDL in patient serum. The development of autoantibodies in the serum can act as an indicator of the presence of OxLDL in the system. Therefore, presence of such

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autoantibodies could serve as an indicator of the development of atherosclerosis.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

- 5 **FIGURE 1.** Chemiluminescent immunoassay to analyze the binding of sera from patients with APS antibodies to cardiolipin exposed to oxygen for various amounts of time.
- FIGURE 2.** Radioimmunosorbent assay (RIA) to analyze the binding of E0 antibodies to LDL oxidized by copper for various amounts of time.
- 10 **FIGURE 3.** Binding of E06 to OxLDL and carrier-linked PC.
- FIGURE 4.** Competition of carrier-linked PC to the binding of OxLDL to E06.
- FIGURE 5.** Inhibition of E06 binding to Cu-OxLDL by both E06 and T15.

- The present invention will be better understood from the following
- 15 detailed description of an exemplary embodiment of the invention, taken in conjunction with the accompanying drawings in which like reference numerals refer to like parts and in which:

### **DETAILED DESCRIPTION OF THE DRAWINGS**

- 20 **FIGURE 1.** Chemiluminescent immunoassay of the binding of sera from APS patients to cardiolipin (CL). CL was rapidly dried in microtiter wells under nitrogen until the ethanol was completely evaporated, and then the wells were exposed to air at room temperature for 0 to 26 hours. A 1:100 dilution of patient or control sera in 3% BSA, 0.27mM EDTA and 20 $\mu$ M  $\beta$ -hydroxytoluene
- 25 (BHT) was incubated with each well for one hour. After washing, the amount of bound antibody was detected by the use of alkaline-phosphatase goat anti-human IgM or IgG and chemiluminescent substrate. Data are expressed as a number of flashes of light counted per 100ms. Each point represents the mean value of at least four different wells (Horroko, 1996).
- 30 **FIGURE 2.** Binding of natural monoclonal antibodies to LDL oxidized to different extents. Native LDL (0 time) was exposed to copper ions (5 $\mu$ M) for indicated periods of time and then used to coat microtiter wells. 50 $\mu$ l of each purified antibody were added per well and antibody binding was detected with <sup>125</sup>I-labeled goat anti-mouse IgM. The time scale is not linear.
- 35 **FIGURE 3.** Binding of E06 and IgA T15 to oxidation specific epitopes of LDL and phosphorocholine. A series of antigens (Cu-OxLDL, POVPC-BSA, PC-



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KLH, PC-BSA, PC-histone, and native LDL) were plated on microtiter wells at indicated concentrations overnight at 4°C. E06 (panel A) or T15 (panel B) were added at a concentration of 5µg/ml followed by alkaline- phosphatase conjugated goat anti-mouse IgM or IgA secondary antibodies. The amount of bound antibody was expressed as RLU/100ms.

**FIGURE 4.** Inhibition of E06 binding to Cu-OxLDL by phosphorocholine as a sodium salt (PC-Cl) or PC-KLH conjugate. Cu-OxLDL (10µg/ml) was plated on a microtiter wells overnight at 4°C. E06 (10µg/ml) was added to wells in the absence or presence of indicated concentrations of competitors and the amount of E06 bound was detected by alkaline-phosphatase conjugated goat anti-mouse IgM. The amount of E06 bound was expressed as the percent of E06 binding to Cu-OxLDL in the absence of competitor. **Inset.** Competition by PC-Cl for the binding of E06 to Cu-OxLDL and E014 to its antigen, MDA-LDL.

**FIGURE 5.** Inhibition of E06 binding to Cu-OxLDL by both E06 and T15. Cu-OxLDL (10 µg/ml) was plated on microtiter wells overnight at 4°C. Biotinylated E06 (10 µg/ml) was added to wells in the absence or presence of indicated concentrations of competitors. The amount of biotinylated E06 bound to CuOxLDL was detected by alkaline phosphatase conjugated NeutrAvidin, and results expressed as percent control of biotinylated E06 binding to the antigen in the absence of competitor. Non-specific mouse IgA was used as an isotype control for T15.

#### **DETAILED DESCRIPTION AND PREFERRED EMBODIMENTS**

The formation of OxLDL is an essential step in the development of atherosclerosis. OxLDL leads to the formation of foam cells that contribute to the formation of fatty streaks and atherosclerotic plaques in the vasculature. OxLDL is also a strong antigen that results in the development of autoantibodies exacerbating the immune response. By monitoring the levels of OxLDL in the blood, one could monitor atherosclerotic disease in a non-invasive, inexpensive manner.

Immunoassays are commonly used to monitor the level of substances in the serum. A number of antibodies have been identified that bind specifically to OxLDL; however, due to the innate instability of OxLDL in all its forms, it is innately problematic as a control or competitor in a commercial immunoassay. The invention is a method for the use of phosphorylcholine

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(PC), either as a salt (e.g. PC-Cl) or linked to a carrier (e.g. KLH, BSA), as a competitor in an immunoassay using the OxLDL specific monoclonal antibody E06. The E06 antibody binds OxLDL and PC with a high avidity (at least  $10^5$ -fold preference of OxLDL vs. non-OxLDL).

5 T15 is a classic antibody to phosphorylcholine (PC), which is the headgroup moiety  $[-PO_4-(CH_2)_2-N-(CH_3)_3]$  of many phospholipids including PAPC. Because the genetic and idiotypic analyses indicated that these E0 antibodies to OxLDL used VH/VL regions that were identical to those of T15, the antigen binding properties of T15, an IgA antibody, were systematically  
10 compared with a representative E0 antibody, E06, an IgM antibody. As expected, E06 bound to Cu-OxLDL and POVPC-BSA, but not to native LDL or MDA-LDL (fig 4A). As previously reported, T15 bound to its known antigen, PC, derivatized onto carrier proteins, BSA or Keyhole limpet hemocyanin (KLH). Similarly to E06, T15 also bound to Cu-OxLDL and  
15 POVPC-BSA, but not to native LDL or MDA-LDL (fig 4B). In addition, akin to the reactivity of T15, E06 bound to the PC conjugates of both PC-BSA and PC-KLH. Neither E06 nor T15 bound native, non-oxidized phospholipid PAPC.

To further investigate the fine binding specificity of E06 to PC, a  
20 competition immunoassay was performed. Fig 5 shows the binding of E06 to Cu-OxLDL was totally inhibited by soluble PC (PC-Cl), as well as by PC-KLH, whereas the binding of another oxidation specific IgM monoclonal autoantibody, E014 to its antigen, MDA-LDL, was unaffected by PC-Cl (fig 5 inset). In analogous experiments, the binding of T15 to Cu-OxLDL was also  
25 inhibited by PC-Cl in a dose dependent manner (data not shown).

The presence of antibodies against OxLDL may be indicative of various disease states including atherosclerosis, acute coronary artery syndrome, kidney dysfunction, diabetes, and preeclampsia. PC-linked to a carrier protein could be coated onto microtiter wells for use in an assay to  
30 detect anti-OxLDL antibodies in patient sera.

The following examples are given to enable those of ordinary skill in the art to more clearly understand and to practice the invention. The examples should not be considered as limiting the scope of the invention, but  
35 merely as illustrative and representative thereof.

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EXAMPLE 1

**Hybridomas and monoclonal antibodies.** Monoclonal antibodies directed against OxLDL specific epitopes were produced from hybridomas generated from ApoE<sup>-/-</sup> mice, which have very high titers of autoantibodies to Cu-OxLDL (WO 99/08109 incorporated herein by reference). In brief, B-lymphocytes from the spleens of two ApoE<sup>-/-</sup> mice, that had not been immunized exogenously, were fused with the P3 x 63Ag8.653.1 myeloma cell line. Hybridomas were screened for binding to model epitopes of OxLDL including Cu-OxLDL and MDA-LDL. Seventeen hybridoma cell lines were isolated by limiting dilution and designated as E0 antibodies, e.g. E01 - E017. All were isotyped as IgM and characterized for binding properties. Antibodies bound to OxLDL vs. non-OxLDL with at least a 10<sup>5</sup>-fold stronger binding. Antibodies were purified from ascites fluid by fast protein liquid chromatography (FPLC).

EXAMPLE 2

**Sequencing of VL and VH genes of hybridoma antibodies.** Total RNA was extracted from 10<sup>7</sup> hybridoma cells using RNA STAT-60 (Tel-Test, Friendswood, TX) using the manufacturer's protocol. cDNA was then synthesized with oligo dT primer using Superscript II cDNA synthesis kit (GIBCO-BRL, Gaithersburg, MD). Each PCR reaction contained 0.5 µg of cDNA template and 1 µg of each oligonucleotide primer. The reactions were supplemented with dNTP, Taq polymerase, and reaction buffer, and amplified under optimized thermal cycler conditions. For the first round of PCR, VH genes were amplified in seven different reaction tubes, each containing one panel of seven sense primers targeted for VH framework 1 subdomain paired with an antisense primer specific for the µ constant region (CH1). The oligonucleotides were designed to amplify greater than 80% of all expressed VH gene rearrangements (Silverman et al., 1998). In preliminary studies, an immunoassay using anti-kappa specific antibody determined that each of the E0 antibodies used a κ light chain. Therefore, V-kappa gene rearrangements were amplified in six separate PCR reactions that included one of two degenerate Vk gene FR1 sense primers and one of three antisense promoters that each target distinct Jk gene sequences.

The PCR products of the murine hybridoma immunoglobulin VH and Vk gene rearrangements were purified following the electrophoretic separation on a 2% agarose gel and then cloned into an appropriate vector.

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Sequences were determined by automated sequencing. Alignments were performed using the EMBL/GenBank and NCBI Blast databases.

### EXAMPLE 3

5     **Chemiluminescent immunoassay.** To coat wells, protein antigens (e.g. LDL, PC linked to carrier proteins) were diluted in PBS (phosphate buffered saline; 10 mM Na-phosphate, 150 mM NaCl, 0.02% Na-azide, pH 7.4) containing 0.27 mM EDTA. 50µl of the solution was applied per well to a 96-well white, round-bottomed MicroFluor (Dynex Technologies, Chantilly, VA)  
10    microtiter plates overnight at 4°C. Phospholipid antigens were dissolved in organic solvents and applied to the plate in a similar fashion. The wells were washed three times with PBS and blocked with PBS containing 1% BSA (bovine serum albumin) for 30 minutes. 50µl of biotinylated EO6 antisera, diluted in PBS-BSA, were added to wells in the presence and absence of  
15    competitors (e.g. phosphorocholine) and then incubated for one hour at room temperature (RT). The wells were washed three times with PBS. Binding of primary antibody to the antigen in the well was detected with alkaline phosphatase-linked goat-anti-mouse immunoglobulin secondary antibodies. Wells were rinsed with water and 25µl of LumiPhos 530 solution (Lumigen)  
20    was added to the wells. In some experiments, E06 antibody was biotinylated and added to antigen coated wells followed by addition of NeutrAvidin (Pierce). The light emission was measured as relative light units (RLU) over a 100 mseconds using a Dynex luminometer (Dynex Technologies)

### EXAMPLE 4

25     **ELISA for OxLDL in patient sera, PC competition assay.** T15 was diluted in PBS containing 0.27 mM EDTA. 50µl of the solution was applied per well to a 96-well white, round-bottomed MicroFluor (Dynex Technologies, Chantilly, VA) microtiter plates overnight at 4°C. The wells were washed  
30    three times with PBS and blocked with PBS containing 1% BSA (bovine serum albumin) for 30 minutes. 50µl of patient sera, diluted in PBS-BSA supplemented with EDTA and BHT or other antioxidants, were added to wells, with various concentrations (0 to 1mM) of PC, and then incubated for one hour at room temperature (RT). The wells were washed three times with  
35    PBS. Anti-apoB antibody, generated in rabbit, diluted in PBS-BSA, was added to wells and incubated for one hour at room temperature. Binding of

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apoB antibody to the LDL in the well was detected with alkaline phosphatase goat-anti-rabbit immunoglobulin secondary antibodies. Wells were rinsed with water and 25µl of LumiPhos 530 solution (Lumigen) was added to the wells. The light emission was measured as relative light units (RLU) over a  
5 100 µseconds using a Dynex luminometer (Dynex Technologies).  
Alternatively, the apo B antibody may be linked directly to alkaline phosphatase for direct detection or to biotin for detection using streptavidin-linked alkaline phosphatase.

10

#### EXAMPLE 5

**ELISA assay for OxLDL in patient sera.** To coat wells, anti-apoB antibody was diluted and the solution was applied to a 96-well microtiter plate overnight at 4°C. Similarly, wells were coated with serial dilutions of KLH-linked PC. Wells were washed three times with PBS and blocked with PBS  
15 containing 1% BSA for 30 minutes. 50 µl of patient sera diluted in PBS with 0.27 mM EDTA containing antioxidants were added to wells containing the anti-apoB antibody and then incubated for one hour at room temperature. The wells containing KLH-linked PC were incubated in 1% BSA in PBS. The wells were washed three times with PBS. Binding of E06 antibody to OxLDL  
20 from patient sera was detected with a horse radish peroxidase goat-anti-mouse immunoglobulin secondary antibody. Wells were rinsed with water and developed using a chromogenic substrate (e.g. 3,3',5,5'-tetramethylbenzidine, o-phenylenediamine dihydrochloride). Colorimetric change was assayed using an automated plate reader.

25

#### EXAMPLE 6

**ELISA for OxLDL in patient sera, competition with E06.** OxLDL was diluted in PBS containing 0.27mM EDTA and antioxidants. 50µl of the solution was applied per well to 96-well, round bottomed MicroFluor (Dynex  
30 Technologies, Chantilly,VA) microtiter plates overnight at 4°C. The wells were washed three times and blocked with PBS containing 1%BSA for 30 minutes. A duplicate set of wells were prepared containing a constant, limiting amount of labeled (e.g. biotinylated) E06 antibody was mixed with various dilutions of patient sera, which may contain OxLDL to compete for  
35 binding to E06, and added immediately to the OxLDL coated well. To generate a standard curve, a set of control wells containing E06 mixed with

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known concentrations of PC were prepared. Plates were incubated for 1 hour at room temperature. In one set of wells, binding of E06 to the OxLDL was detected using streptavidin-linked-alkaline phosphatase. To ensure constant binding of the OxLDL to the plates, the second set of wells are reacted with an anti-apoB100 antibody. Wells were rinsed with water and 25µl of LumiPhos 530 solution (Lumigen) was added to the wells. The light emission was measured as RLU over 100 ms.

#### EXAMPLE 7

10 **Assay for presence of OxLDL antibodies in patient serum.** To coat wells, BSA- or KLH-linked PC was diluted in PBS containing 0.27 mM EDTA. 50µl of the solution was applied per well to a 96-well white, round-bottomed MicroFluor (Dynex Technologies, Chantilly, VA) microtiter plates overnight at 4°C. The wells were washed three times with PBS and blocked with PBS  
15 containing 1% BSA (bovine serum albumin) for 30 minutes. 50µl of patient sera, diluted in PBS-BSA, were added to wells, optionally with competitors, and then incubated for one hour at room temperature (RT). The wells were washed three times with PBS. Binding of antibody from patient sera to PC was detected with alkaline phosphatase goat-anti-mouse immunoglobulin  
20 secondary antibodies. Wells were rinsed with water and 25µl of LumiPhos 530 solution (Lumigen) was added to the wells. The light emission was measured as relative light units (RLU) over a 100 mseconds using a Dynex luminometer (Dynex Technologies)

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10 Although an exemplary embodiment of the invention has been described above by way of example only, it will be understood by those skilled in the field that modifications may be made to the disclosed embodiment without departing from the scope of the invention, which is defined by the appended claims.

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**I CLAIM:**

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### CLAIMS

1. A method for standardization of an immunological assay to detect oxidized low density lipoproteins (OxLDL) in a sample comprising:
  - (a) contacting a sample containing OxLDL with an antibody that has a high affinity for both OxLDL and phosphorylcholine (PC),
  - (b) allowing time for the antibody to bind the OxLDL,
  - (c) determining the amount of OxLDL bound to the antibody, and
  - (d) using PC to generate a standard curve to quantitate the amount of antibody bound.
2. A method as in claim 1, wherein the antibody is E06 or T15.
3. A method as in claim 2, wherein the antibody is a monoclonal antibody, polyclonal antibody or an single chain antibody variable region fragment (scVf).
4. A method as in claim 1, wherein the antibody is linked to a detectable marker.
5. A method as in claim 1, wherein the antibody is detected by the use of a second antibody labeled with a detectable marker.
6. A method as in claim 1, wherein the PC is a salt or is linked to a carrier protein.
7. A method for standardization of an immunological assay to detect oxidized low density lipoproteins (OxLDL) in a sample comprising:
  - (a) contacting a sample containing OxLDL with an antibody that has a high affinity for both OxLDL and phosphorylcholine (PC),
  - (b) allowing time for the antibody to bind the OxLDL
  - (c) determining the amount of OxLDL bound to the antibody, and
  - (d) using PC as a competitor for the binding of the OxLDL to the antibody to determine the amount of OxLDL present.
8. A method as in claim 7, wherein the antibody is E06 or T15.



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9. A method as in claim 8, wherein the antibody is a monoclonal antibody, polyclonal antibody or an single chain antibody variable region fragment (scVf).
10. A method as in claim 7, wherein the PC is a salt or is linked to a carrier protein.
11. A method for the detection of antibodies that bind to oxidized low density lipoprotein (OxLDL) comprising:
  - (a) contacting an antibody containing solution with phosphorylcholine (PC) linked to a carrier protein,
  - (b) allowing time for the antibody to bind the OxLDL, and
  - (c) detecting the amount of antibody bound.
12. A method as in claim 11, wherein the source of the antibodies is patient sera.
13. A method as in claim 11, wherein the presence of antibodies that bind PC is indicative of a disease state.
14. A method as in claim 11, wherein the antibodies bound to PC are detected by the use of a second antibody labeled with a detectable marker.

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FIGURE 1

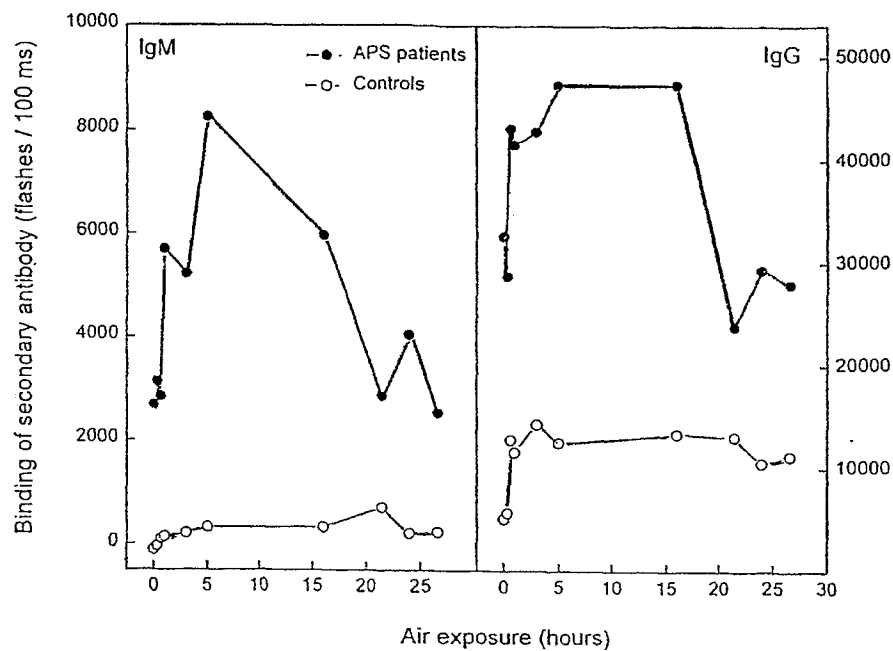
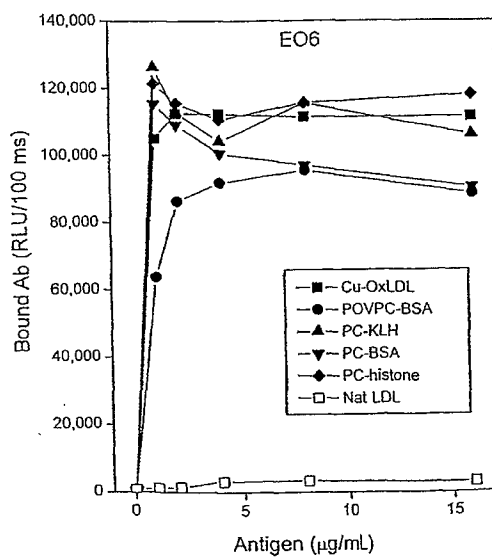


FIGURE 3

a



b

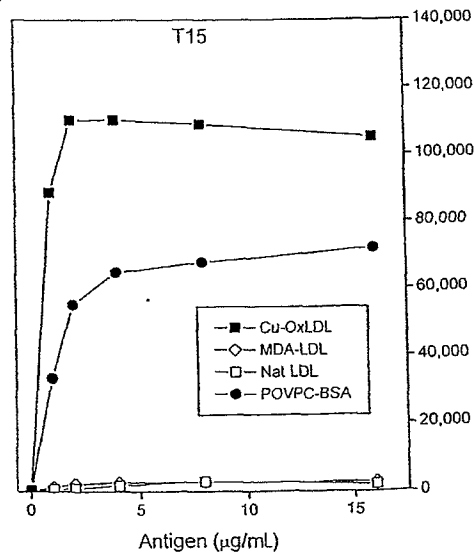
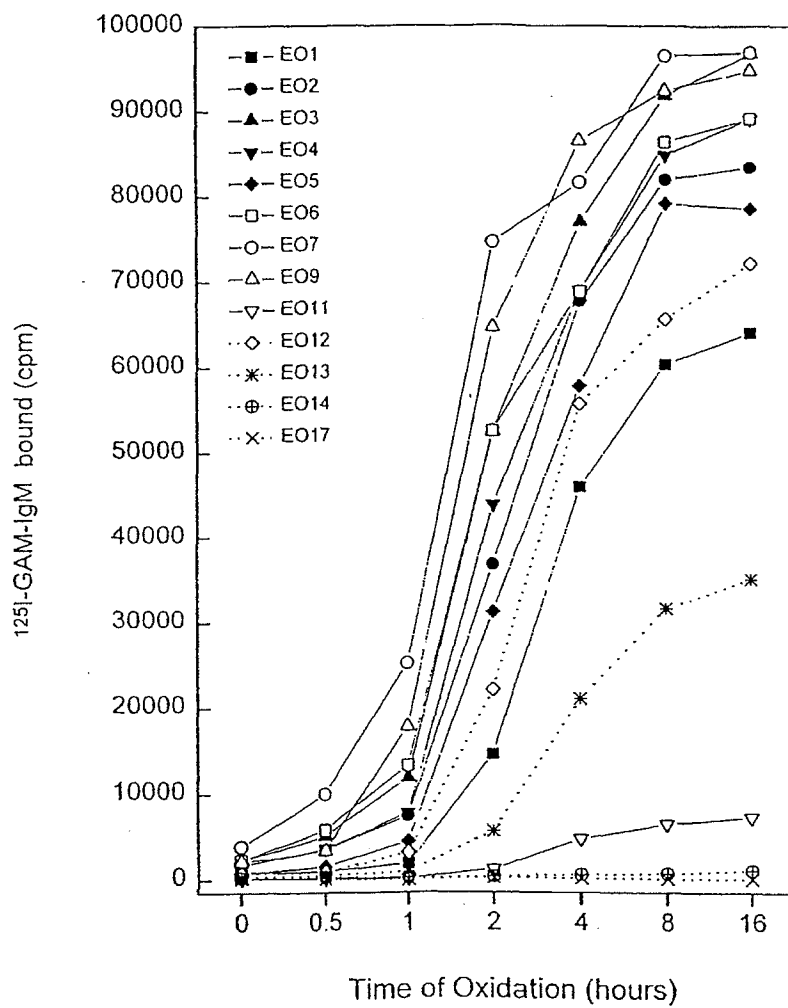


FIGURE 2



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FIGURE 4

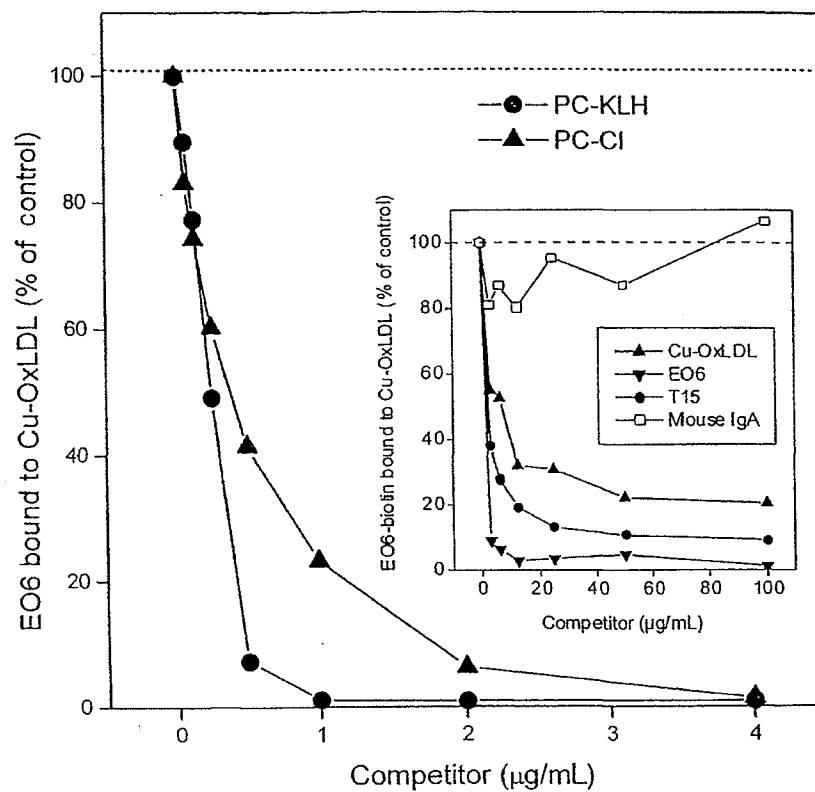


FIGURE 5

